Topical siRNA delivery to the cornea and anterior eye by hybrid silicon-lipid nanoparticles

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Paulina Baran-Rachwalska [a,b], Nissim Torabi-Pour [a], Flavia Maria Sutera [a], Mukhtar Ahmed [a], Keith Thomas [b], M. Andrew Nesbit [b], Michael Welsh [a], C.B. Tara Moore [a,b,1]* and Suzanne R. Saffie-Siebert [a,1]

[a] *SiSaf Ltd, Surrey Research Park, Guildford GU2 7RE, United Kingdom*

[b] *Biomedical Sciences Research Institute, University of Ulster, Coleraine BT52 1SA, United Kingdom*

Author Note

[1] These authors share senior authorship: C.B. Tara Moore and Suzanne R. Saffie-Siebert

* Correspondence should be addressed at:

Biomedical Sciences Research Institute, University of Ulster, Cromore Road, Coleraine BT52 1SA, Northern Ireland, United Kingdom; E-mail address: *tara.moore@ulster.ac.uk* (C.B.T. Moore)

Abstract

The major unmet need and crucial challenge hampering the exciting potential of RNAi therapeutics in ophthalmology is to find an effective, safe and non-invasive means of delivering siRNA to the cornea. Although all tissues of the eye are accessible by injection, topical application is preferable for the frequent treatment regimen that would be necessary for siRNA-induced gene silencing. However, the ocular surface is one of the more complex biological barriers for drug delivery due to the combined effect of short contact time, tear dilution and poor corneal cell penetration. Using nanotechnology to overcome the challenges, we developed a unique silicon-based delivery platform for ocular delivery of siRNA. This biocompatible hybrid of porous silicon nanoparticles and lipids has demonstrated an ability to bind nucleic acid and deliver functional siRNA to corneal cells both *in vitro* and *in vivo*. Potent transfection of human corneal epithelial cells with siRNA-ProSilic® formulation was followed by a successful downregulation of reporter protein expression. Moreover, siRNA complexed with this silicon-based hybrid and applied *in vivo* topically to mice eyes penetrated across all cornea layers and resulted in a significant reduction of the targeted protein expression in corneal epithelium. In terms of siRNA loading capacity, system versatility, and potency of action, ProSilic provides unique attributes as a biodegradable delivery platform for therapeutic oligonucleotides.

Keywords:

silicon, nanoparticles, siRNA, drug delivery, topical, cornea, ocular, gene therapy, ProSilic

1. Introduction

Personalised medicine in the form of RNA interference (RNAi) is a promising therapeutic approach for a wide range of genetic disorders. Utilization of small interfering RNA (siRNA) molecules and a naturally occurring RNAi pathway leading to homology-dependent degradation of a particular messenger RNA and specific gene downregulation has shown great potential in treating cancer, cardiovascular diseases, neurodegenerative diseases, inflammatory conditions, viral infections and ocular diseases [1]. Gene therapy represents an ideal strategy for treatment of diseases of the cornea – with some of them being rare and having no effective treatments beyond corneal transplant available. According to the World Health Organisation, corneal blindness is the fourth most common cause of blindness globally and is one of the major causes of visual deficiency after cataract, glaucoma and age-related macular degeneration [2]. Although eye tissues are accessible by injection, a non-invasive topical drug administration is considered preferable as a frequent patient-friendly treatment regimen because invasive routes of administration can be associated with complications such as intraocular infection, pain, and discomfort to the patient which result in poor patient compliance [3,4]. However, the intrinsic physical barriers, efficient drug clearance mechanisms and other complexities of ocular tissues pose a significant challenge to ocular nucleic acid delivery. The ocular surface is one of the more complex biological barriers for drug delivery due to the combined effect of short contact time, the presence of tear film and poor permeability of the cornea. One of the greater challenges to topical treatment of eye disorders is the structure of the cornea, which is adapted to form an effective barrier against fluid loss and pathogen penetration. The highly organized, multilayered, corneal epithelium, connected by intra-cellular tight junctions, and the hydrophilic stroma which lies underneath make the transport of drugs, into and across it, very difficult. Alternative absorption through the conjunctival pathway is considered non-productive due to

high vascularization of the conjunctiva, because the systemic circulation removes most of the drug before it can enter the inner ocular tissues [5]. Also a large portion of the topically applied dose may be absorbed systematically through the naso-lacrimal duct drainage thereby lowering ocular bioavailability [6]. Additionally, drug molecules are diluted on the pre-corneal tear film, which itself has a rapid turnover rate. This, together with the blinking reflex, severely limits the residence time of topically administered drugs in the pre-corneal space and reduces their ocular bioavailability to below 5% of the instilled drug dose [7].

While most drugs exhibit difficulties in overcoming the eye-associated barriers, delivery of large hydrophilic charged RNA molecules is particularly challenging. Although there are examples of direct instillation of siRNA onto the ocular surface in animal experiments and clinical trials [8–11], the limited efficiencies of the treatments have often forced researchers to use high siRNA doses or a frequently repeated treatment regimen. Moreover, naked siRNA oligonucleotides do not readily cross the anionic cell membrane due to their high molecular weight, large size and negatively charged phosphate backbone. Additionally, siRNA in its native form is relatively unstable *in vivo*, prone to degradation by ribonucleases, which are ubiquitous both in the extracellular and the intracellular environment. Furthermore, exogenous RNA may stimulate innate immune responses, cause cellular toxicity or induce undesirable off-target effects [12,13]. Chemical modifications in siRNAs have provided means to overcome some of the problems associated with administration of synthetic nucleic acids *in vivo* [14], but the real potential of siRNA therapeutics would be realized only if an effective intracellular delivery to target cells is achieved. Conjugation of siRNA with lipids or ligands such as antibodies, aptamers, peptides or small molecules can facilitate cellular uptake and alter siRNA biodistribution [15]. Nucleic acid encapsulation within nanoparticles is potentially much more effective, because it could allow for the combination of all desired properties: siRNA protection, improved cell and

tissue penetration, controlled release of drug, and targeted delivery [16–18]. However, the design of a topically applied nanosystem effective in delivery of siRNA into corneal cells is made difficult due to distinctive anatomical and physiological barriers of the eye, as mentioned above. Often highly potent *in vitro* formulations fail to overcome challenges related to *in vivo* ocular delivery. For example, trials with commercially available transfection agents have been shown to be unsuccessful for *in vivo* delivery of siRNA when applied topically to intact [19] or debrided cornea [9].

Here we demonstrate for the first time that a non-viral vector hybrid silicon-lipid nanoparticle delivery system, the Prosilic®, composed of biodegradable silicon nanoparticles (SiNPs), lipids and amino acids is able to complex with the siRNA and deliver it to the cornea following non-invasive topical administration. The delivery system strongly enhances the bioavailability of functional siRNA within the cytoplasm and mediates targeted gene expression reduction. In this unique delivery system reported within, the surface of the SiNPs is associated with amino acids and lipid agents which form surface linkages with the silicon nanoparticles to modify the rate of silicon hydrolysis, facilitating the biodegradation of silicon into its beneficial and bioactive form, orthosilicic acid, without polymerization [20].

2. Materials and Methods

2.1. Preparation and characterization of silicon nanoparticles

Si-NPs were sourced from a number of suppliers (silicon nanopowder #633097, <100 nm by TEM, purity ⩾ 98% from Sigma-Aldrich, UK; and nano silicon powder #100GS, <100 nm by laser diffraction, ICP purity 99%, BET surface area >34 m^2/g from Zhengzhou Dongyao Nano Materials, China). These were assessed in preliminary studies and determined to be suitable for use, with the Zhengzhou Dongyao material being used for the remainder of the studies. Si-NPs were preliminary subjected to a slow evaporation technique, after methanol rinsing, until

dryness reached [21–23]. Afterwards the SiNPs have been analysed for determining Si content, available surface area, particle size and residual solvent prior to further formulation process. Inductively coupled plasma mass spectrometry (ICP-MS) analysis was performed to determine the concentration of ^{28}Si in the SiNPs following microwave-assisted acid digestion [24]. SiNPs fine powder was digested in a mixture of HCl and HNO_3 (3:1, aqua regia) in a microwave oven Multiwave Pro (Anton Paar). The digestion was done at a maximal pressure of 40 bars and at a maximal temperature of 210°C. For each digestion cycle, a blank and a Certified Reference Material (Sigma Aldrich, UK) were added to respectively control the cleanliness of the tubes and determine the Si extraction recovery. After the digestion process, the samples were diluted to 5 mg/mL by adding ultra-pure water, centrifuged and filtered through 0.2 µm filters. The ICP-MS analysis was performed using Shimadzu ICPE-9800 and the content of ^{28}Si was determined with a calibration curve after applying the dilution factor and the recovery factor obtained from the certified reference material. Surface area analysis was performed by gas sorption using Quantachrome Nova 2200e and calculated from the Brunauer–Emmett–Teller (BET) theory. Before the measurements, the samples were degassed at 350°C overnight. Transmission electron microscopy (TEM) was used to visually examine the size and structure of SiNPs. Particle suspension was diluted to around 0.01% in deionized water and a drop of sample was dried at reduced temperature under vacuum over a carbon-coated copper grid. The samples were then observed at an accelerating voltage of 100 kV under FEI Talos L120C G2 TEM.

2.2. Preparation of silicon-based hybrid nanoparticle formulation

The ProSilic delivery system was prepared with various compositions according to SiSaf patent US9132083B2 [20]. All chemicals used in the preparation were purchased from Sigma-Aldrich (UK) unless otherwise stated. In general, formulations were prepared starting with a lipid thin film generated by means of solvent evaporation and hydrated with SiNPs

aqueous solution as follows. In particular, phospholipids such as soybean phospatidylcholine (PC) and dioleoyl L-α-phosphaltitylethanolamine (DOPE) (Lipoid GmbH, Germany), and cationic lipids such as stearylamine and 3β-[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) (Sigma-Aldrich, UK) were solubilized in a sufficient quantity of chloroform at concentrations in region of 5 to 10 mg/mL, and then subjected to solvent evaporation by rotary evaporation (Heidolph Laborota 4001). A PC:stearylamine mass ratio of 90:10 was used to formulate a first ProSilic variant prototype, named ProSilic-PS91G formulation, while a DOPE:DC-chol:stearylamine mass ratio of 60:30:10 was used to formulate a second Prosilic variant prototype, the ProSilic-DSC613G formulation. SiNPs (Zhengzhou Dongyao), pretreated as described above, were dispersed at 1 mg/mL in aqueous solution containing 0.5% glycine using sonication. The silicon mixture was then used to hydrate the lipid thin film (at 1:4 SiNP to overall lipid weight ratio), and the final silicon-lipid preparation was lyophilized. Prior to analysis samples were rehydrated with nuclease-free water (Thermo Scientific, USA), and incubated at room temperature for 1 h unless otherwise described.

2.3. Residual solvent analysis

All samples were analyzed for residual solvents in accordance with USP method <467> [25]. For headspace gas chromatography, 1 mg of the sample was weighed into a headspace vial, then 1mL deionised water was added and the sample vial was capped with a magnetic lid. The analysis was performed using Shimadzu GC-2030 connected to an FID detector, with a Shimadzu RTx-624 column. Autosampler/Headspace conditions: incubation 80°C for 60min, syringe 90°C, 250rpm, prepurge time 30sec, injection flow rate 10mL/min. Injector conditions: hydrogen as gas carrier, 140°C, split ratio 5:1, carrier gas linear speed 35cm/sec. Oven temperature: baseline at 40°C for 12 min, then ramp/rate 16.7°C/min for 12 minutes then hold at 240°C for 12 minutes. FID temperature 250°C, nitrogen flow

30mL/min, air flow 400mL/min. For blank measurement deionized water was used, and standard curves for methanol (50-5000ppm) and chloroform (2-80ppm) quantification were prepared from the certified reference material purchased from Sigma Aldrich, properly diluted with deionized water for reaching desired concentration.

2.4. Loading of siRNA on ProSilic nanoparticles

In the studies, two different siRNAs were used: siLUC targeting *Firefly* luciferase reporter gene (sense: 5′–CGA-CAA-GCC-UGG-CGC-AGU-AdTdT–3′, antisense: 5′– UAC-UGC-GCC-AGG-CUU-GUC-GdTdT–3′) and a non-specific siRNA (NSC4) with an inverted bacterial ß-galactosidase sequence (sense: 5′–UAG-CGA-CUA-AAC-ACA-UCA-AdTdT–3′, antisense: 5′–UUG-AUG-UGU-UUA-GUC-GCU-AdTdT–3′) which served as a negative control to establish that any decrease in target gene expression is related to a sequence-specific RNAi event [8,26–30]. Both siRNAs were designed as 21-mers with a central 19 bp duplex region and symmetric dTdT dinucleotide overhangs on each 3'end. These were chemically synthesized and annealed by Eurogentec (Belgium). For nucleic acid complexation with both ProSilic formulations, siRNA dissolved in nuclease-free water was added to the aqueous solution of nanoparticles and incubated at room temperature for 60 minutes prior to further analysis. To examine the best complexation ratio, siRNA was mixed with the nanoparticles at different w/w ratios and analysed by gel electrophoresis. Additionally, complexation efficiency was determined using high speed centrifugation and UV spectrophotometry as described below. An optimal loading ratio of 1:11 was chosen for all further experiments.

2.5.Gel retardation assay

To assess the formation of complexes between the siRNA and ProSilic variants, nanoparticles dispersed in nuclease-free water were combined with siRNA at various loading ratios (i.e. 1:2.75, 1:5.5, 1:11 or 1:22 w/w of siRNA to ProSilic) and analyzed by

electrophoresis on a 1% agarose gel (UltraPure Agarose, ThermoFisher, UK) in 0.5 × TBE (Tris-Borate-EDTA, ThermoFisher) for 40 min at 100 V. Equal amount of 150ng siRNA was loaded into each well. The gel was visualized using the Gel Logic 100 Imaging System (Kodak).

2.6. Assessment of siRNA entrapment efficiency

Complexation efficiency was determined as a fraction of entrapped siRNA compared to the total amount of nucleic acid added. ProSilic preparations were dispersed in nuclease-free water, combined with siRNA at 1:2.75, 1:5.5, 1:11 or 1:22 w/w ratio and diluted to a final siRNA concentration of 100 μg/mL. Unloaded siRNA was used as a control. Samples were centrifuged at 21,000 rcf for 30 min (Mikro 200R centrifuge, Hettich, UK), and the unbound siRNA in the supernatant was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). The amount of entrapped siRNA was calculated from the difference of siRNA concentration in supernatant solutions of test samples and unloaded siRNA control (an initial amount added). The measurements were made in triplicate, and the values expressed as mean ± SD. The results were also used to estimate loading capacity by calculating the amount of siRNA bound to 1mg of ProSilic nanoparticles.

2.7. Particle size and surface charge measurement

Mean hydrodynamic particle size and zeta-potential of siRNA-loaded and empty ProSilic was determined using ZetaSizer Nano ZS (Malvern Panalytical, UK). All measurements were undertaken in nuclease-free water and particles loaded with siRNA were prepared at 1:11 w/w complexation ratio as used in further studies. Samples were diluted with nuclease-free water to yield count rate in a range of 100-400 kcps. In parallel, lipoplexes composed of siRNA and Lipofectamine RNAiMAX (ThermoFisher Scientific, UK), a well-known lipid-based carrier specifically designed for siRNA delivery, were prepared in nuclease-free water in accordance with manufacturer's instructions and analyzed for particle

size and zeta-potential. All measurements were made at 25°C in triplicate and reported as mean ± SD.

2.8. Cell culture

Spontaneously immortalized human corneal epithelial (HCE-S) cell line was kindly gifted by J.T. Daniels (Institute of Ophthalmology, University College London, UK) [31]. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher, UK), incubated under 5% CO_2 at 37°C and passaged following standard laboratory procedures. Twenty-four hours prior to transfection, HCE-S cells were seeded at 7×10^4 cells per well on 12-well plates for flow cytometry study or 7×10^3 cells per well in 96-well plates for dual luciferase assay.

2.9. Transfection efficiency assessment by flow cytometry

To study cellular uptake of siRNA complexed with ProSilic, a fluorescent oligonucleotide duplex known as an indicator of siRNA transfection (FAM-labelled siGLO, Dharmacon, UK) was complexed with nanoparticles at 1:11 loading ratio and added to HCE-S cells at 0.1μM final concentration in DMEM. Transfection with Lipofectamine RNAiMAX at 0.1μM siGLO and 0.1μM naked siGLO were used as positive and negative controls, respectively. The cells were incubated at 37°C for 24 h, then detached from the plate surface with 0.25% trypsin-EDTA (Gibco, UK), washed with PBS and resuspended in Pharmingen™ Stain Buffer (BD Biosciences, USA) supplemented with propidium iodide (Invitrogen, UK) to allow identification of dead cells. Flow cytometry analysis was performed using a Gallios™ Flow Cytometer with Klauza software (both Beckman Coulter, UK).

2.10. Evaluation of siRNA delivery in vitro

Knockdown of reporter gene expression was assessed with a dual luciferase assay, modified as previously reported [8,27–30], in which expression of *Firefly* luciferase, the siRNA target, is normalized to *Renilla* luciferase expression as an internal control of cell

transfection. HCE-S cells were firstly transfected with both *Firefly* and *Renilla* luciferase reporter plasmids [26] using Lipofectamine 2000 (Thermo Fisher, Invitrogen, UK) according to manufacturer's protocol. Then 24 h later, cells were washed with PBS and treated with ProSilic formulations complexed with luciferase-specific siLUC or non-specific control NSC4. Samples were prepared at 1:11 loading ratio as described above, diluted with complete medium to 0.1μM final siRNA concentration and added to the cells which were then incubated at 37ºC for 48 h. As a positive control, cells were transfected with 0.1 μM siLUC or NSC4 complexed with Lipofectamine RNAiMAX in accordance with manufacturer's recommendation. Seven replicates were studied for each condition. The Dual-Luciferase Reporter Assay (Promega, UK) was used to measure the luciferase expression 48 hours after transfection. The assay was used according to manufacturer's instructions. Briefly, the medium was removed, and cells were washed with PBS before replacement with Passive Lysis Buffer (Promega, UK). Culture plates were shaken on a plate shaker for 15 minutes to ensure cells were fully lysed, before the activities of both *Firefly* and *Renilla* luciferases were measured sequentially using a microplate luminometer (LUMIstar OPTIMA, BMG Labtech, UK). The values obtained for *Firefly* were normalized to *Renilla* activity, and results were expressed as a percentage of the relative luciferase activity measured in negative control (100%).

2.11. Live animal imaging

Animals were used for the following experiments in accordance with the UK Animal Welfare Act with ethical approval by the Home Office (Scotland) and the DHSSPS (Northern Ireland). The experiments to assess delivery of fluorescent siRNA (DY-547-labeled siGLO, Dharmacon, UK) to the cornea were performed on wild-type C57BL/6 mice. To assess siRNA bioavailability and silencing activity of formulations, a reporter knock-in mouse line (Krt12+/luc2) was used, with the expression of *Firefly* luciferase specifically in the cornea

epithelium (under the control of the endogenous *Krt12* promoter) [8]. This animal model was developed on a C57BL/6 background as previously reported and provides a reliable model for *in vivo* evaluation of siRNA delivery methods using reporter gene expression monitoring. For *in vivo* imaging, mice were anaesthetized using 1.5–2% isoflurane (Abbott Laboratories Ltd., UK) in ~1.5 L/min flow of oxygen. Fluorescence of siGLO was detected with a Xenogen IVIS Spectrum with LivingImage 3.2 software (both Perkin Elmer, UK) using DsRed filter combination (excitation 535 nm, emission 570 nm) at determined time points following topical application. To measure luciferase reporter gene expression, luciferin (30 mg/mL D-luciferin potassium salt; Gold Biotechnology, USA) mixed 1:1 w/w with Viscotears gel (Novartis, UK) was dropped onto the eye of anaesthetized mouse immediately prior to imaging. Bioluminescence readouts were taken by IVIS Spectrum over a period of approximately 10 min and quantified using the LivingImage software after ensuring the signal remained stable within an acquisition time. For signal intensity quantification, a region of interest (ROI) was selected separately for each eye keeping ROIs parameters (size and shape) constant throughout experiments, and values expressed as the right/left eye ratio (RE/LE) using split body control measurement regime as previously described [8,32].

2.12. In vivo siRNA treatment

Experiments were performed using a split body control by comparing the treatment under a test, in one eye, with a negative control in the other eye of the same animal. During treatment procedure mice were anesthetized as described above. ProSilic formulations containing 25μM siRNA complexed at 1:11 loading ratio were prepared and applied topically as a drop to the intact cornea in a total volume of 4 μL per eye. After application, the mouse was kept anesthetized for a further 15 min to allow absorption and maximize uptake. Following treatment, fluorescence and luminescence experiments were performed as described below.

2.13. Assessment of siRNA penetration to the cornea

To investigate delivery of siRNA to the cornea, *in vivo* fluorescence study was performed on wild-type mice using eye drops containing siGLO. The fluorescent siRNA-ProSilic formulation was applied on top of the right eye, whereas same amount of naked siGLO was applied topically to the left eye of each mouse as a control. Fluorescence live imaging was acquired with IVIS Spectrum at 15min (i.e. immediately after treatment procedure) and 3, 6, and 24 h following siGLO application, and signal intensity normalized to background fluorescence measured prior treatment (i.e. untreated eyes) and quantified as described earlier. After measurements taken at either 3h or 24h, mice were sacrificed, and the eyes were enucleated, fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, submerged in PolyFreeze (Sigma-Aldrich, UK), and immediately frozen at -80ºC. Five-micrometer sections were cut using a cryostat (CM 1850, Leica), mounted on APES-coated slides (3-aminopropyltriethoxysilane, Sigma Aldrich, UK) with DAPI-containing mounting medium (DAPI I, Vysis, USA) and fluorescence was visualized with AxioScope A1 microscope equipped with a 20×/40× N Archoplan lens on an AxioCam MRc camera (Carl Zeiss, Germany).

2.14. Assessment of siRNA-mediated gene silencing

Luciferase reporter mice (n = 7) were used to determine bioavailability of siRNA in the cornea after topical delivery with ProSilic-DSC613G as a leading candidate. In a split body control experiment, luciferase-targeting siLUC complexed with ProSilic formulation was applied topically as a 4µl drop to the intact cornea of right eye (RE) in anesthetized mice, whereas the left eye (LE) was treated accordingly with nonspecific NSC4-ProSilic as negative control. Treatment was repeated daily for 8 consecutive days, with *in vivo* ocular luminescence measurements taken approximately 4-5 hours later. The effect of treatment on luciferase reporter gene expression was determined by measurement of luciferase

bioluminescent activity (as described above) daily throughout treatment regimen, and for a further 8 days after cessation of treatment for monitoring a wash-out period. Baseline luminescence was defined for each experimental animal by monitoring ocular luciferase activity at 24-hour intervals for 4 days prior to treatment. The relative RE/LE luciferase bioluminescent activity was quantified using the IVIS LivingImage software and plotted as an average value ± standard deviation.

2.15. Evaluation of in vivo safety (biocompatibility)

In order to monitor the occurrence of toxic effects, mice eyes were examined by an ophthalmic surgeon at various time points during treatment and for a further 8 days after treatment withdrawal. After the final measurement of luminescence, animals were sacrificed and eyes enucleated for tissue histology carried out as previously described [8]. Histological slices (5µm) were obtained from each eye and stained with haematoxylin and eosin (H&E, both solutions from Sigma-Aldrich, UK) to assess general morphology of the cornea.

2.16. Statistical analysis

Data are presented as the means ± standard deviation and are representative for at least 3 independent measurements, unless stated otherwise. Statistical significance was assessed with a one or two-way ANOVA followed by the Tukey's HSD *post hoc* test at 95% confidence level. For *in vitro* dual luciferase assay, a two-tailed Student's t test was performed for each formulation separately to analyze knockdown level (siLUC vs NSC4 control). For the *in vivo* luciferase experiment, the statistical analysis was done by comparing the average RE/LE ratio for all seven mice in the first 4 days before the beginning of the treatment (set as baseline) with the RE/LE ratios measured on the following days. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, USA).

3. Results

3.1. SiNPs characterization

SiNPs, after being subjected to methanolic rinsing and slow evaporation process, were characterized for determining a content of silicon, particle size, zeta-potential and specific surface area. ICP analysis revealed purity above 98% with other metal traces below 2% of the overall mass. TEM images confirmed spherical shape and the particle size within the range of 100nm. The mean surface area as measured by BET was approx. 34 m^2/g. Zeta-potential of SiNPs was determined to be −30.4 mV ± 3.3 and accounted a typical negative charge of porous silicon material that relates to the presence of hydroxyl groups on SiNPs surfaces [33]. After formulating the silicon-based couriers, samples were analyzed for residual solvents, and both chloroform and methanol level were determined to be below the minimum concentration limit (60ppm and 3000ppm, respectively) [25].

3.2. Characterization of the silicon-based siRNA delivery system

Two ProSilic variants were formulated through surface functionalization of silicon nanoparticles with cationic lipids commonly used in nucleic acid delivery, stearylamine and DC-cholesterol, which resulted in hybrid particles with similar hydrodynamic sizes of ~350nm and comparative positive zeta potential values (Table 1). Complexation of cationic ProSilic formulations with siRNA studied by gel electrophoresis showed full entrapment of nucleic acid with a minimum 1:11 loading ratio (Figure 1A). The percentage of complexed siRNA for varying w/w ratios was determined by spectrophotometry and calculated from the differences of siRNA amount added to the carrier and the concentration of siRNA in solutions after particle separation (Figure 1B). Higher siRNA entrapment efficiency was observed for complexation with ProSilic-DSC613G containing cationic cholesterol derivative compared to ProSilic-PS91G functionalized only with stearylamine as cationic lipid. ProSilic-DSC613G formulation showed siRNA loading capacity in a range of 6.3-8.7 nmol per 1mg

nanoparticles, whereas ProSilic-PS91G variant showed to be capable of entrapping 2.3-3.4 nmol siRNA per 1mg particles. Following siRNA loading studies, a fixed 1:11 complexation ratio was chosen for all further experiments.

As the physicochemical properties of nanoparticles play an important role in drug delivery, particle size and surface charge of siRNA-loaded complexes were determined (Table 1). ProSilic-DSC613G appeared similar in dimensions and zeta potential when empty and loaded carrier were compared, whereas ProSilic-PS91G showed an increased mean particle size and negative surface charge when complexed with siRNA suggesting an absorption of nucleic acid molecules on the surface of hybrid particles in addition to internal siRNA entrapment. Both ProSilic formulations considered in this study were compared to the gold standard in gene silencing, Lipofectamine™ RNAiMAX, a commercially available lipid-based carrier specifically designed for siRNA delivery. ZetaSizer analysis of empty and siRNA-loaded RNAiMAX also showed an increase in particle size and an inversion of surface charge from positive to negative values after complexation with nucleic acid.

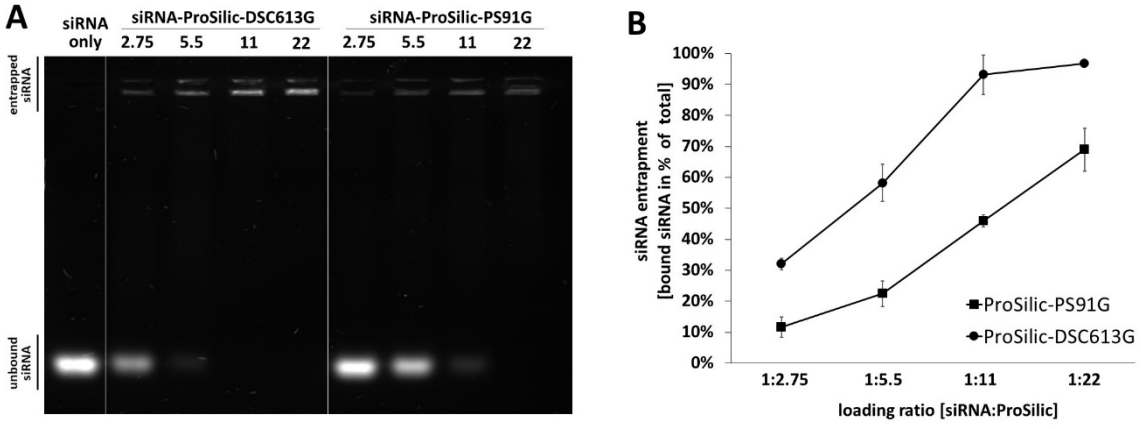

Figure 1. The optimization of siRNA-ProSilic complexation ratio. (A) Gel retardation assay in which uncomplexed siRNA migrated through 1% agarose gel, while the siRNA bound by ProSilic remained immobilized within the wells. Line 1 (from left side) was loaded with unformulated siRNA (control). Line 2-5 and 6-9 were loaded with siRNA formulated

with ProSilic-DSC613G or ProSilic-PS91G variants, respectively, at increasing ratio from 1:2.75 to 1:22 (w/w). (B) Entrapment efficiency expressed as a fraction of bound siRNA compared to the total amount of nucleic acid added. The siRNA was complexed with ProSilic-DSC613G or ProSilic-PS91G at increasing loading ratio from 1:2.75 to 1:22 (w/w). Three replicates were studied for each condition and means ± standard deviation, are presented.

Table 1. ***Average particle size and zeta potential of tested samples.***

	Sample	Particle size [nm]	Zeta potential [mV]
ProSilic-PS91G	empty	351 ± 32	+36.6 ± 1.0
	with siRNA	462 ± 57	−39.7 ± 0.6
ProSilic-DSC613G	empty	361 ± 39	+40.9 ± 0.9
	with siRNA	397 ± 44	+34.9 ± 0.7
RNAiMAX	empty	133 ± 3	+23.2 ± 12.0
	with siRNA	212 ± 32	−57.0 ± 7.1

Note: Measurements were undertaken in nuclease-free water. Data represent mean ± standard deviation (n=3). SiNPs particles alone having size of 100nm in average showed negative zeta potential of −30.4 mV ± 3.3.

3.3. Evaluation of in vitro siRNA delivery to corneal cells

For initial *in vitro* screening, human epithelial corneal epithelial cell line (HCE-S) was used to evaluate ProSilic efficacy in cellular transfection and its potential cytotoxicity. Transfection efficiency was quantified by flow cytometry analysis performed 24 hours after treatment with fluorescent oligonucleotide duplex loaded to carrier system (Figure 2A). ProSilic-DSC613G showed 55±2% and ProSilic-PS91G 65±6% of FAM-positive cells in comparison to 84±1% observed for RNAiMAX reagent. The post-transfection cell viability assessed based on live/dead staining with propidium iodide (PI), a common indicator of membrane disintegration, showed ProSilic formulations were well tolerated by corneal epithelial cells (Figure 2B) in contrary to Lipofectamine transfection agent, which in spite of being highly potent for the delivery of exogenous nucleic acids into cells *in vitro*, is not

suitable for clinical applications. Over 50% of cells transfected with Lipofectamine RNAiMAX were shown to have damaged membranes as indicated by staining with PI dye whereas more than 86% and 98% intact cells were observed after treatment with ProSilic formulations.

Next, bioavailability of siRNA was evaluated in gene expression studies using dual luciferase reporter assay. After treatment of HCE-S cells with 0.1μM siLUC complexed with the ProSilic nanoparticles (Figure 2C), a knockdown of 46±5% ($p<0.01$, siLUC vs NSC4 control) and 38±8% ($p<0.01$) was achieved for ProSilic-PS91G and ProSilic-DCS613G variants, respectively, whereas siLUC transfected with RNAiMAX reduced luciferase reporter gene expression by 66±9% ($p<0.001$). Taken together, ProSilic delivery system demonstrated up to 70% potency of commercial siRNA transfection reagent while being safe and well-tolerated by the cells.

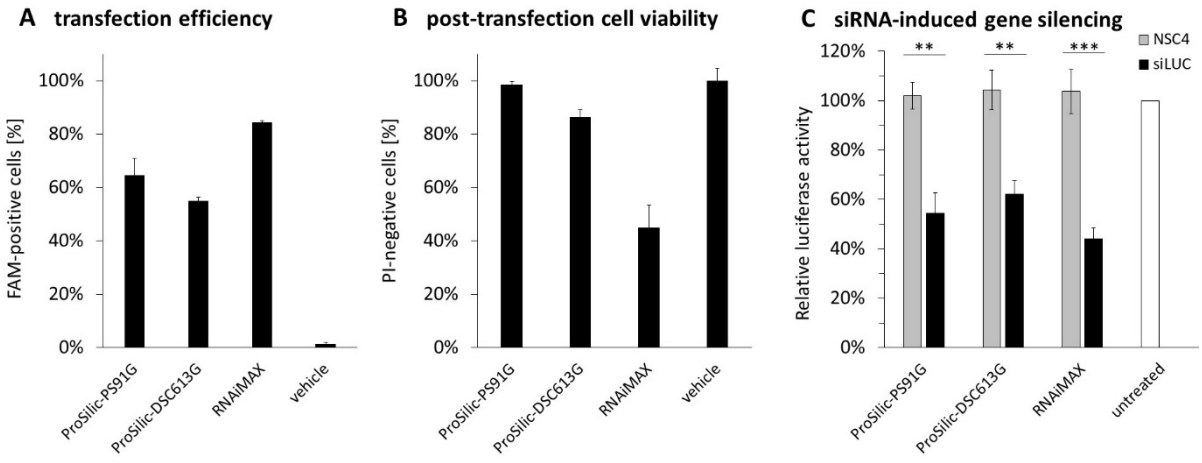

***Figure 2*. In vitro siRNA delivery to human corneal epithelial cells**. Transfection efficiency (A) and post-transfection cell viability (B) assessed in HCE-S cells by flow cytometry analysis after 24-hour incubation with 0.1μM fluorescent FAM-siGLO complexed with either ProSilic-PS91G, ProSilic-DSC613G or Lipofectamine RNAiMAX. Transfection efficiency is expressed as a percentage of FAM-positive cells in total gated. Cytotoxicity of formulations expressed as a percentage of PI-negative cells was normalized to negative control (vehicle). Three replicates were studied for each condition and means ± standard

deviation are presented. (C) *In vitro* luciferase gene knockdown assessed on HCE-S cells treated with 0.1μM siRNA: a luciferase-targeting siRNA (siLUC, black bar) or a negative control nonspecific siRNA (NSC4, grey bar), complexed with either ProSilic-PS91G, ProSilic-DSC613G or Lipofectamine RNAiMAX (positive control). Relative luciferase activity was measured 48 hours after siRNA treatment using dual luciferase reporter gene expression assay and expressed as a percentage of the negative control (untreated cells, white bar). Data shown is a mean of n=6 replicates ± standard error.

3.4. Evaluation of in vivo ocular siRNA delivery by topical ProSilic administration

Following the demonstration of successful siRNA delivery and gene knockdown *in vitro*, the two ProSilic variants were evaluated *in vivo* for topical administration to the anterior eye. Firstly, ProSilic-PS91G and ProSilic-DCS613G variants were complexed with fluorescent siGLO and applied as an eye drop to wild-type mice following a unilateral procedure with the naked siGlo control being instilled in the opposite eye. The ocular fluorescence was monitored for up to 24 h using an *in vivo* imaging system (Figure 3A). The first measurement was performed 15 min after administration when the mouse was still under anaesthesia after treatment, and the following measurements were repeated at 3, 6, and 24 h post-administration. Although an equal amount of 100pmol siGLO was applied topically to each eye, the highest fluorescence intensity measured 15 min later was observed for the treatment with ProSilic-DSC613G, subtly less for ProSilic-PS91G, and two times less for naked siGLO ($p<0.05$) that indicated an increased ocular surface adhesion of formulated drug and an improved residence time in comparison with naked oligonucleotide. Three hours later, *in vivo* fluorescence signal decreased 3-fold in siGLO-ProSilic-DSC613G-treated eyes, whereas it had returned to baseline levels for siGLO-ProSilic-PS91G and unformulated siGLO eye drops due to active ocular clearance mechanisms. Although further gradual decrease of *in vivo* signal intensity was observed, fluorescence in the eyes treated with siGLO-ProSilic-DSC613G persisted for up to 24 h and was significantly higher than in eyes

treated with the unformulated naked siGLO at all the time points ($p<0.01$ at 3 h and 6 h, and $p<0.05$ at 24 h). This suggested an effective uptake of topically administered siRNA drug formulated with ProSilic. However, to verify nanoparticle permeation into the tissue, distribution of the siRNA throughout the cornea layers was investigated by fluorescence microscopy of post-treatment corneal cryosections (Figure 3B). Red siGLO fluorescence was detected throughout all corneal layers in all ProSilic-treated sections of eyes collected 3 hours after eye drops application, whereas no fluorescence above background was observed in the naked siGLO control. Twenty-four hours after the administration of siRNA formulations, the fluorescence was only observed in the cornea sections treated with ProSilic-DSC613G, thus considered as leading candidate for further evaluation.

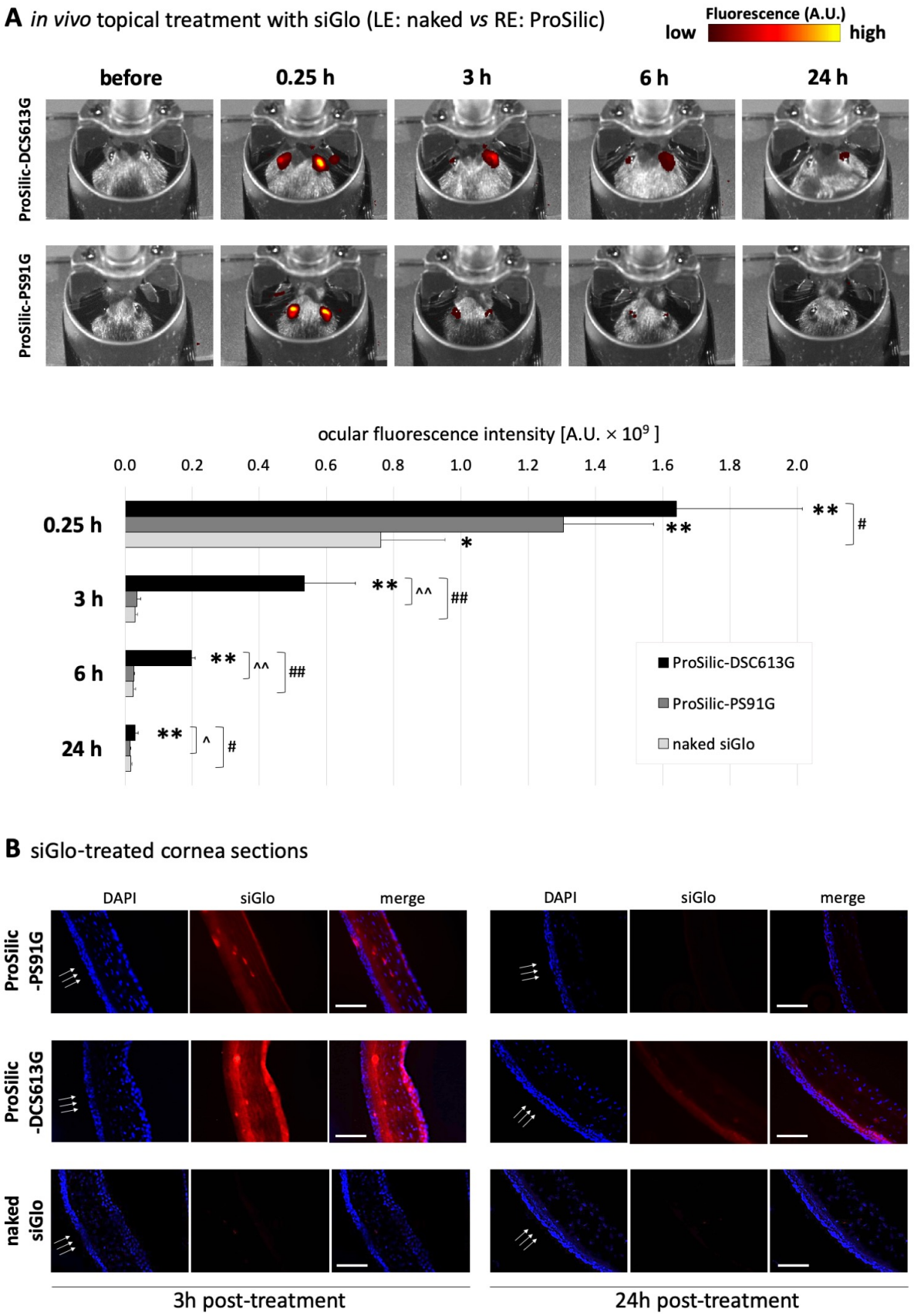

***Figure 3. In vivo* topical siRNA delivery**. Wild-type mice were treated with an equal amount of naked or formulated fluorescent siRNA applied topically as eye drops and live imaging

was carried out at 15 minutes and 3, 6, and 24 hours after treatment (A). Experiment was performed with a split body control in which siGLO loaded to ProSilic (right eye, RE) was compared with naked siGLO (left eye, LE). The red and yellow pseudo-colours represent the intensity of the fluorescence as indicated (not the colour of the fluorescent signal). Graph displays ocular fluorescence intensity, and values are expressed a mean (± standard error) of n = 3-6 independent measurements. *, $p<0.05$; **, $p<0.01$ *vs* untreated control (baseline); ^, $p<0.05$; ^^, $p<0.01$ ProSilic-PS91G *vs* naked siGLO, and $^{\#}$, $p<0.05$; $^{\#\#}$, $p<0.01$ ProSilic-DSC613G *vs* naked siGLO by two-way ANOVA. (B) Corneal 5μm cryosections of the mouse eyes, collected and fixed after 3 or 24 hours post-treatment, were imaged using fluorescence microscope after mounting with DAPI-containing medium (scale bar, 100μm). The nuclei are stained blue, whereas the red fluorescence represents siGLO, and the arrows indicate outer surface.

Following *in vivo* uptake studies, the siRNA delivery with leading ProSilic-DSC613G candidate was further investigated in functional assays using a murine reporter model with luciferase expression confined exclusively to the corneal epithelium. Prior to *in vivo* treatment, basal corneal luciferase activity in reporter mice was quantified every 24 h for 4 days to confirm a consistent right-to-left ratio for a split body control experiment. ProSilic-DSC613G complexed with siLUC or control siRNA was applied topically as eye drops to opposite eyes of the same animal 8 times in daily intervals, and corneal luciferase expression was evaluated every day by live animal imaging throughout treatment regimen and over following 8 days (Figure 4). A reduced luciferase expression was observed within 24 h of treatment initiation, and a maximal inhibition (41% ±13, $p<0.001$) was achieved at day 11. A significant gene silencing effect persisted throughout entire treatment regimen and continued for 4 days after termination of treatment. As expected, the reduced ocular bioluminescence level gradually returned to baseline after treatment withdrawal which indicated a successful recovery from gene repression. Importantly, gross examination of treated eyes and daily visual inspection of animals following topical treatment revealed no adverse effects of eye

drops. At no time point were any signs of swelling, edema or inflammation noted. H&E staining of treated eye sections did not show any alteration of the corneal layers, nor evidence of inflammation or cellular infiltration (Figure 4D), suggesting ProSilic formulation was well-tolerated *in vivo*.

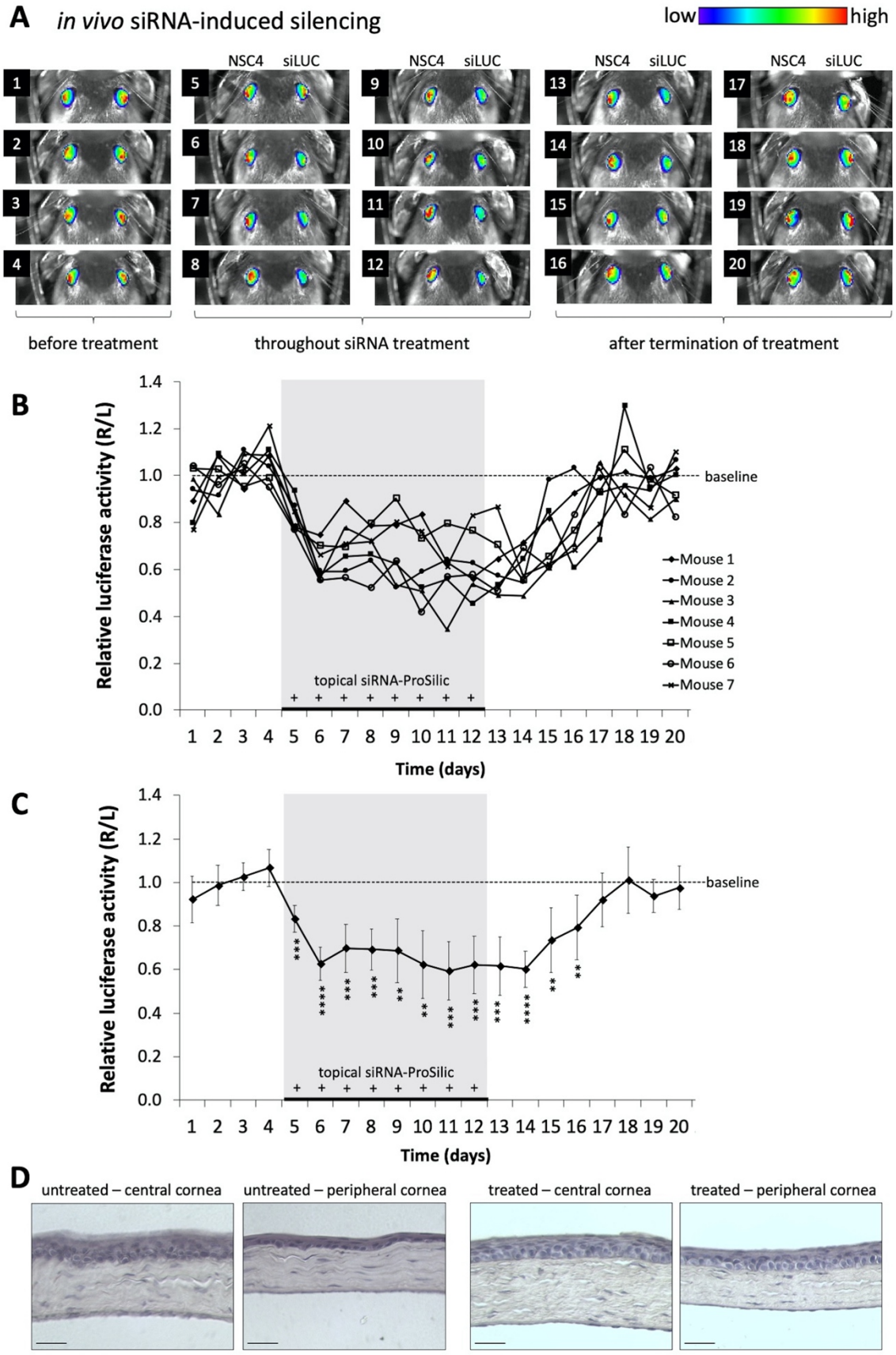

***Figure 4*. In vivo siRNA-mediated gene silencing in reporter mice after topical delivery**. Reporter mice expressing luciferase specifically in the corneal epithelium were treated with

eye drops containing siLUC complexed with ProSilic-DSC613G applied to right eye (RE). As a negative control non-specific siRNA (NSC4) complexed with same ProSilic-DSC613G was applied topically to left eye (LE). The bioluminescent luciferase activity was measured using Xenogen IVIS Spectrum system daily throughout treatment regimen, and for a further 8 days after cessation of treatment for monitoring a wash-out period. Baseline luminescence was defined for each experimental animal by monitoring ocular luciferase activity at 24-hour intervals for 4 days prior to treatment. (A) Representative daily images of one reporter mice during monitoring the effect of the topical treatment with siRNA complexed with ProSilic-DSC613G. (B) Graph showing individual bioluminescence readouts performed on seven reporter mice using split body control, expressed as the right eye/left eye ratio (RE/LE). (C) The effect of topical treatment on luciferase reporter gene expression determined for a treatment group (n = 7) expressed as an average value of relative RE/LE luciferase bioluminescent activity (siLUC vs NSC4) ± standard deviation. Significant knockdown of luciferase with siLUC-ProSilic was observed from day 5 to day 16: $^{**}p < 0.01$, $^{***}p < 0.001$. (D) Representative sections of H&E stained central and peripheral cornea of eyes treated with eye drops containing siRNA with ProSilic-DSC613G collected from mice after the termination of experiment.

4. Discussion

The potency and versatility of siRNA therapeutics has attracted drug developers who have envisioned that RNAi technology will help to overcome the limitations of classical small molecule drugs in targeting certain disease-associated proteins [34]. However, the full potential of therapeutic RNAs remains unfulfilled due to a compelling deficiency in effective delivery systems. In this paper, we present evidence indicating that our hybrid silicon-lipid nanoparticle delivery system exhibits characteristics that enable efficient encapsulation and intracellular delivery of siRNA, and prove it effective in topical delivery to the eye which will allow development of a non-invasive treatment of ocular pathologies.

A nucleic acid carrier is a required component of RNAi-based therapy because treatment with unmodified naked siRNA does not result in effective knockdown of the target gene [35]. Large, highly charged siRNA molecules cannot pass freely through the plasma

membrane unless conjugated or complexed with delivery agents. Besides chemical modifications to increase stability, target specificity and potency [14,36], various molecules can be conjugated to the 5' or 3' ends of siRNA strands to facilitate cellular uptake [15]. An incorporation of ligands such as peptides, aptamers or cholesterol can allow for cell specific targeting and improve distribution and cellular uptake of siRNA bioconjugates [15,37]. However, the choice of modification pattern depends on the specific siRNA sequence, route of delivery and type of application, and the effect of the modification is generally difficult to predict, with empirical testing needed to ensure that the resulting molecule is still effective and retains the desired properties [38]. Also, there are concerns about degradation of these artificially altered siRNA molecules which may generate metabolites with unsafe or unwanted reactivity [12]. Moreover, an uptake of siRNA by fluid-phase endocytosis should not be confused with the delivery of functional siRNA into the cytoplasm [35]. Endosomal entrapment and lysosomal degradation represents one of the key effect-limiting factors in siRNA delivery [8,13,39]. In view of these circumstances, the encapsulation of unmodified or partially modified siRNA within nanoparticles is potentially beneficial because, in addition to siRNA protection, better tissue penetration and controlled release can be achieved [16–18]. Also retargeting the drug by a replacement of siRNA sequence without changing *in vivo* pharmacokinetics raises the potential for truly 'programmable' therapeutics [34], expediting progress of novel siRNAs to clinical application.

Nanotechnology has revolutionized the field of ocular drug delivery by improving the bioavailability of therapeutic molecules at ocular tissues [4,16,40,41]. Nanoparticles with desired surface characteristics get internalized by cells which, in turn, enables penetration of the entrapped therapeutic agents across the eye. The ProSilic carrier system reported herein is a hybrid delivery technology that combines elemental silicon with lipids and amino acids, that demonstrates an ability to bind siRNA and deliver it effectively into the cytoplasm

which, in turn, leads to targeted downregulation of gene mRNA expression. The use of porous silicon and porous silica as a carrier for the delivery of nucleic acid has been reported previously, however, the importance of the degradation products of such carrier systems has not received sufficient attention [42–47]. In ProSilic technology, through association of a stabilizing agent to the surface of silicon nanoparticles, the rate of silicon hydrolysis can be controlled, such that the SiNPs dissolve to the orthosilicic acid [20], which is a major bioavailable form of silicon for both humans and animals and an essential micromineral for cellular health [48]. Additionally, the presence of lipids in the composition has a beneficial effect on the surface properties of the nanoparticles, enhancing their interaction with cell membrane, improving loading of siRNA and controlling the release of cargo. Cationic lipids promote the interactions between the carrier and the loaded nucleic acid by providing a positive zeta potential and helping to condense anionic siRNA molecules to be incorporated into the system [49]. The role of electrostatic interactions in the loading of charged molecules into the porous core has been previously demonstrated. Liu *et al*. showed that a fusion of positively charged liposomes with negatively charged mesoporous silica particles can be used to load these particles with negatively charged molecules at high concentrations [37]. In our studies, a simple co-incubation of siRNA with hybrid silicon-lipid delivery system allowed for efficient complexation of nucleic acid with the nanoparticles, and showed siRNA loading capacity in a range of 2.3-8.7 μmol per gram of ProSilic which compares favourably with other delivery systems [43,51–53]. It was observed that the type of surface functionalization of silicon particles with different lipids influenced the siRNA loading efficiency and resulted in various zeta potential values for siRNA-loaded particles. As shown by our experimental results, both tested formulations were positively charged before siRNA complexation, whereas an addition of siRNA to the nanoparticles inverted the positive zeta potential of ProSilic-PS91G but had no significant effect on ProSilic-DCS613G net charge. Considering a

lower loading capacity was observed for ProSilic-PS91G, a portion of siRNA cargo is likely to have associated on the outer surface of nanoparticles and exposed negatively charged phosphate groups whereas ProSilic-DCS613G allowed greater encapsulation of siRNA and expressed cationic zeta potential. For comparison, lipofectamine also showed an inversion of surface charge from positive to negative values upon siRNA complexation, consistent with results reported previously [54]. Nevertheless, while all formulation samples were shown to be effective in siRNA delivery to corneal epithelial cells *in vitro*, it was observed that the ProSilic formulations demonstrated much greater cellular compatibility compared to RNAiMAX.

Adjustment of the lipid composition allows not only control of the cargo content, but also optimization of the physicochemical properties of nanoparticles for specific biological applications. It is commonly known that particle size, shape and surface charge are key determinants in cellular uptake of nanoparticles, however zeta-potential is considered to have the highest impact on the internalization [55] because most charged functional groups are responsible for active nanoparticle interaction with cells [56]. Positively charged complexes are well-known to bind to the negatively charged cell membrane components, such as proteoglycans, and translocate across the plasma membrane. There has been also evidence of uptake of negatively charged particles despite their unfavourable interaction with the negatively charged domains of cell membrane [55]. Thus, variation of the particle surface properties could potentially control their binding to the tissue but also influence intracellular fate by targeting to certain cellular compartments, such as lysosomes, mitochondria, or cytoplasm [48,49]. In ocular drug delivery, a positive charge can facilitate effective adhesion to the cornea epithelial surface, and account for a strong interaction with the negatively charged eye mucosa and anionic mucins present in the tear film, prolonging the residence time and enhancing the drug bioavailability in the internal tissue of the eye [57]. In agreement

with previous reports [58,59], our *in vivo* data indicated that net positive charge carried by the transfection complex can facilitate an effective penetration of the nano-system across ocular tissues.

The results presented here are the first example of successful topical delivery of siRNA to the cornea using biocompatible and biodegradable nanoparticles. ProSilic has been shown to be capable of complexing the siRNA, delivering it into the cells of all corneal layers, and releasing functional siRNA into the cytoplasm resulting in specific knockdown of mRNA expression as demonstrated in this transgenic mouse model. While RNAi-induced gene silencing within the cornea, at levels sufficient for therapeutic application has proven to be extremely challenging, our achievements have exceeded *in vivo* gene silencing using non-invasive topical siRNA delivery reported to date [8]. With further optimization of the platform and siRNA design a greater gene knockdown in the cornea should be achieved, currently accomplishable only by intrastromal injections with chemically modified siRNAs. Such invasive methods as ocular injection are considered unsuitable for repeated and long-term application in the ophthalmology clinic. Furthermore, nanoparticles can offer a sustained drug release and increased residence time of entrapped siRNA molecules in ocular tissues which can help to reduce the frequency of drug administration for the effective treatment of ocular diseases. The RNAi approach is a particularly attractive tool for a selective gene therapy treatment of a wide spectrum of corneal diseases, including corneal dystrophies such as Meesmann Epithelial Corneal Dystrophy [26], TGFBI-associated corneal dystrophies [28] or Fuchs Endothelial Corneal Dystrophy [60], and inflammatory diseases [2,11]. Corneal dystrophies are heritable diseases that affect the transparency or shape of the cornea which can lead to progressive vision loss and eventually blindness [61]. Symptoms can start to show from an early age, or can be induced due to surgical interventions, such as elective refractive laser correction surgery, cataract surgery or lamellar or penetrating keratoplasty, designed to treat

the pathology [62,63]. Given the perpetual shortage of corneal donors and the limited graft survival rates, surgical treatment for these genetic disorders is often reserved as a last resort and offered once the patient has endured a lifetime of pain and deteriorating eyesight, to near visual loss with significant impact upon quality of life [64–66]. Therefore, the development of a mutation-specific, gene silencing topical eye-drop treatment would be a major advance in how these ophthalmology genetic disorders are treated and managed – offering an early intervention to prevent a lifetime of suffering and vision loss with all the socioeconomic and healthcare intervention costs. However, key to the success of this therapeutic strategy is efficient siRNA delivery to the targeted cells.

5. Conclusions

In summary, to achieve optimal gene silencing efficiency for RNAi effectors, a successful delivery of functional RNA oligonucleotides is critical. Our biodegradable and bioavailable silicon-lipid hybrid delivery system, ProSilic®, demonstrates the ability to bind siRNA at high concentrations and deliver the cargo to the cells both *in vitro* and *in vivo*. Furthermore, a proof of concept study using an animal corneal reporter model revealed a significant knockdown of gene expression after non-invasive topical eye drop administration of siRNA complexed with the ProSilic® system. Due to the versatility of this delivery system, we anticipate that it could be further tailored to efficiently deliver alternative gene therapy agents such as CRISPR Cas complexes [64,67–69] and custom made to target other cells / tissues / organs in a multitude of applications. A local and topical gene therapy approach as outlined within would overcome many of the concerns surrounding systemic delivery and the need for repeated injections into the eye. Most importantly progress made by our team and others investigating the potential for gene therapy for corneal disease will allow this research to move toward the clinical setting and ultimately reach the patient.

Author Contributions

Paulina Baran-Rachwalska: Methodology, Investigation, Validation, Formal analysis, Writing - Original Draft preparation, Visualization. **Nissim Torabi-Pour**: Conceptualization, Methodology, Resources, Supervision. **Flavia Maria Sutera**: Resources, Writing - Review and Editing. **Mukhtar Ahmed**: Methodology, Investigation, Validation, Resources, Supervision. **Keith Thomas**: Methodology, Investigation. **M. Andrew Nesbit**: Conceptualization, Methodology, Validation, Resources, Writing - Review and Editing, Project Administration, Supervision. **Michael Welsh**: Conceptualization, Methodology, Validation, Writing - Review and Editing, Project Administration, Funding acquisition, Supervision. **C.B.Tara Moore**: Conceptualization, Methodology, Validation, Resources, Writing - Review and Editing, Project Administration, Funding acquisition, Supervision. **Suzanne R. Saffie-Siebert**: Conceptualization, Methodology, Writing - Review and Editing, Project Administration, Supervision.

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Conflicts of Interest

S.R. Saffie-Siebert is the inventor of ProSilic® technology. P. Baran-Rachwalska, N. Torabi-Pour, F.M. Sutera, M. Ahmed and M. Welsh are employed by SiSaf Ltd. C.B.T. Moore acts as consultant for SiSaf Ltd. M.A. Nesbit and K. Thomas declares no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

[1] J. Guo, K.A. Fisher, R. Darcy, J.F. Cryan, C. O'Driscoll, Therapeutic targeting in the silent era: advances in non-viral siRNA delivery, Mol. Biosyst. (2010). https://doi.org/10.1039/c001050m.

[2] E. Di Iorio, V. Barbaro, G. Alvisi, M. Trevisan, S. Ferrari, G. Masi, P. Nespeca, H. Ghassabian, D. Ponzin, G. Palù, New Frontiers of Corneal Gene Therapy, Hum. Gene Ther. 30 (2019) 923–945. https://doi.org/10.1089/hum.2019.026.

[3] R. Gaudana, H.K. Ananthula, A. Parenky, A.K. Mitra, Ocular Drug Delivery, AAPS J. 12 (2010) 348–360. https://doi.org/10.1208/s12248-010-9183-3.

[4] D.A. Srinivasarao, G. Lohiya, D.S. Katti, Fundamentals, challenges, and nanomedicine-based solutions for ocular diseases, Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology. 11 (2019) 1–26. https://doi.org/10.1002/wnan.1548.

[5] V. Agrahari, A. Mandal, V. Agrahari, H.M. Trinh, M. Joseph, A. Ray, H. Hadji, R. Mitra, D. Pal, A.K. Mitra, A comprehensive insight on ocular pharmacokinetics, Drug Deliv. Transl. Res. 6 (2016) 735–754. https://doi.org/10.1007/s13346-016-0339-2.

[6] E. Sánchez-López, M. Espina, S. Doktorovova, E.B. Souto, M.L. García, Lipid nanoparticles (SLN, NLC): Overcoming the anatomical and physiological barriers of the eye – Part II - Ocular drug-loaded lipid nanoparticles, Eur. J. Pharm. Biopharm. 110 (2017) 58–69. https://doi.org/10.1016/j.ejpb.2016.10.013.

[7] S. Reimondez-Troitiño, N. Csaba, M.J. Alonso, M. De La Fuente, Nanotherapies for the treatment of ocular diseases, Eur. J. Pharm. Biopharm. 95 (2015) 279–293. https://doi.org/10.1016/j.ejpb.2015.02.019.

[8] D. Schiroli, M.J. Gómara, E. Maurizi, S.D. Atkinson, L. Mairs, K.A. Christie, D.F. Cobice, C.M. McCrudden, M.A. Nesbit, I. Haro, T. Moore, Effective In Vivo Topical

Delivery of siRNA and Gene Silencing in Intact Corneal Epithelium Using a Modified Cell-Penetrating Peptide, Mol. Ther. - Nucleic Acids. 17 (2019) 891–906. https://doi.org/10.1016/j.omtn.2019.07.017.

[9] Z. Li, F. Duan, L. Lin, Q. Huang, K. Wu, A new approach of delivering siRNA to the cornea and its application for inhibiting herpes simplex keratitis., Curr. Mol. Med. 14 (2014) 1215–25. https://doi.org/CMM-EPUB-62964 [pii].

[10] A. Martin-Gil, M.J.P. de Lara, A. Crooke, C. Santano, A. Peral, J. Pintor, Silencing of P2Y 2 receptors reduces intraocular pressure in New Zealand rabbits, Br. J. Pharmacol. 165 (2012) 1163–1172. https://doi.org/10.1111/j.1476-5381.2011.01586.x.

[11] J. Moreno-Montañés, A.M. Bleau, A.I. Jimenez, Tivanisiran, a novel siRNA for the treatment of dry eye disease, Expert Opin. Investig. Drugs. 27 (2018) 421–426. https://doi.org/10.1080/13543784.2018.1457647.

[12] M.S. Shim, Y.J. Kwon, Efficient and targeted delivery of siRNA in vivo, FEBS J. 277 (2010) 4814–4827. https://doi.org/10.1111/j.1742-4658.2010.07904.x.

[13] J. Wang, Z. Lu, M.G. Wientjes, J.L.-S. Au, Delivery of siRNA Therapeutics: Barriers and Carriers, AAPS J. 12 (2010) 492–503. https://doi.org/10.1208/s12248-010-9210-4.

[14] M.A. Behlke, Chemical modification of siRNAs for in vivo use, Oligonucleotides. 18 (2008) 305–319. https://doi.org/10.1089/oli.2008.0164.

[15] I. V. Chernikov, V. V. Vlassov, E.L. Chernolovskaya, Current development of siRNA bioconjugates: From research to the clinic, Front. Pharmacol. 10 (2019). https://doi.org/10.3389/fphar.2019.00444.

[16] M.A. Kamaleddin, Nano-ophthalmology: Applications and considerations, Nanomedicine Nanotechnology, Biol. Med. 13 (2017) 1459–1472. https://doi.org/10.1016/j.nano.2017.02.007.

[17] S.D. Jayasena, Designer siRNAs to overcome the challenges from the RNAi pathway.,

J. RNAi Gene Silencing. 2 (2005) 109–17. http://www.ncbi.nlm.nih.gov/pubmed/19771212.

[18] K. Tiemann, J.J. Rossi, RNAi-based therapeutics–current status, challenges and prospects, EMBO Mol. Med. 1 (2009) 142–151. https://doi.org/10.1002/emmm.200900023.

[19] R.P. Wilkes, D.A. Ward, K.M. Newkirk, J.K. Adams, S.A. Kania, Evaluation of delivery agents used for introduction of small interfering RNAs into feline corneal cells, Am. J. Vet. Res. 74 (2013) 243–247. https://doi.org/10.2460/ajvr.74.2.243.

[20] R. Saffie-Siebert, Delivery System Comprising A Silicon-Containing Material, U.S. Pat. No. 9,132,083, 2015. http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&p=1&u=%2Fnetahtml%2FPTO%2Fsearch-bool.html&r=1&f=G&l=50&co1=AND&d=PTXT&s1=%22sisaf+ltd%22.ASNM.&OS=AN/.

[21] L.T. Canham, A.G. Cullis, C. Pickering, O.D. Dosser, T.I. Cox, T.P. Lynch, Luminescent anodized silicon aerocrystal networks prepared by supercritical drying, Nature. 368 (1994) 133–135. https://doi.org/10.1038/368133a0.

[22] V. Pellegrini, F. Fuso, G. Lorenzi, M. Allegrini, A. Diligenti, A. Nannini, G. Pennelli, Improved optical emission of porous silicon with different postanodization processes, Appl. Phys. Lett. 67 (1995) 1084. https://doi.org/10.1063/1.114457.

[23] D. Bellet, L. Canham, Controlled drying: The key to better quality porous semiconductors, Adv. Mater. 10 (1998) 487–490. https://doi.org/10.1002/(SICI)1521-4095(199804)10:6<487_H::AID-ADMA487>3.0.CO;2-T.

[24] D. Bossert, D.A. Urban, M. Maceroni, L. Ackermann-Hirschi, L. Haeni, P. Yajan, M. Spuch-Calvar, B. Rothen-Rutishauser, L. Rodriguez-Lorenzo, A. Petri-Fink, F. Schwab, A hydrofluoric acid-free method to dissolve and quantify silica nanoparticles

in aqueous and solid matrices, Sci. Rep. 9 (2019) 1–12. https://doi.org/10.1038/s41598-019-44128-z.

[25] <467> Residual Solvents, in: United States Pharmacop. Natl. Formul., The United States Pharmacopeial Convention, 2019. https://www.uspnf.com/sites/default/files/usp_pdf/EN/USPNF/revisions/gc-467-residual-solvents-ira-20190927.pdf.

[26] H. Liao, A.D. Irvine, C.J. MacEwen, K.H. Weed, L. Porter, L.D. Corden, A.B. Gibson, J.E. Moore, F.J.D. Smith, W.H.I. McLean, C.B.T. Moore, Development of allele-specific therapeutic siRNA in Meesmann epithelial corneal dystrophy, PLoS One. 6 (2011). https://doi.org/10.1371/journal.pone.0028582.

[27] S.D. Atkinson, V.E. McGilligan, H. Liao, I. Szeverenyi, F.J.D. Smith, C.B. Tara Moore, W.H. Irwin McLean, Development of Allele-Specific Therapeutic siRNA for Keratin 5 Mutations in Epidermolysis Bullosa Simplex, J. Invest. Dermatol. 131 (2011) 2079–2086. https://doi.org/10.1038/jid.2011.169.

[28] D.G. Courtney, S.D. Atkinson, J.E. Moore, E. Maurizi, C. Serafini, G. Pellegrini, G.C. Black, F.D. Manson, G.H.F. Yam, C.J. MacEwen, E.H.A. Allen, W.H.I. McLean, C.B.T. Moore, Development of Allele-Specific Gene-Silencing siRNAs for TGFBI Arg124Cys in Lattice Corneal Dystrophy Type I, Investig. Opthalmology Vis. Sci. 55 (2014) 977. https://doi.org/10.1167/iovs.13-13279.

[29] E.H.A. Allen, S.D. Atkinson, H. Liao, J.E. Moore, D.M. Leslie Pedrioli, F.J.D. Smith, W.H.I. McLean, C.B.T. Moore, Allele-Specific siRNA Silencing for the Common Keratin 12 Founder Mutation in Meesmann Epithelial Corneal Dystrophy, Investig. Opthalmology Vis. Sci. 54 (2013) 494. https://doi.org/10.1167/iovs.12-10528.

[30] D.G. Courtney, S.D. Atkinson, E.H.A. Allen, J.E. Moore, C.P. Walsh, D.M.L. Pedrioli, C.J. MacEwen, G. Pellegrini, E. Maurizi, C. Serafini, M. Fantacci, H. Liao, A.D.

Irvine, W.H. McIrwin Lean, C.B. Tara Moore, SiRNA silencing of the mutant keratin 12 allele in corneal limbal epithelial cells grown from patients with Meesmann's epithelial corneal dystrophy, Investig. Ophthalmol. Vis. Sci. 55 (2014) 3352–3360. https://doi.org/10.1167/iovs.13-12957.

[31] M. Notara, J.T. Daniels, Characterisation and functional features of a spontaneously immortalised human corneal epithelial cell line with progenitor-like characteristics, Brain Res. Bull. 81 (2010) 279–286. https://doi.org/10.1016/j.brainresbull.2009.08.009.

[32] V. Hegde, R.P. Hickerson, S. Nainamalai, P.A. Campbell, F.J.D. Smith, W.H.I. McLean, D.M. Leslie Pedrioli, In vivo gene silencing following non-invasive siRNA delivery into the skin using a novel topical formulation, J. Control. Release. 196 (2014) 355–362. https://doi.org/10.1016/j.jconrel.2014.10.022.

[33] L.A. Osminkina, M.B. Gongalsky, Porous silicon suspensions and colloids, Handb. Porous Silicon Second Ed. 1–2 (2018) 227–245. https://doi.org/10.1007/978-3-319-71381-6_125.

[34] R.L. Setten, J.J. Rossi, S. Han, The current state and future directions of RNAi-based therapeutics, Nat. Rev. Drug Discov. 18 (2019) 421–446. https://doi.org/10.1038/s41573-019-0017-4.

[35] D.B. Rozema, D.L. Lewis, siRNA delivery technologies for mammalian systems, TARGETS. 2 (2003) 253–260. https://doi.org/10.1016/S1477-3627(03)02381-X.

[36] S.H. Ku, S.D. Jo, Y.K. Lee, K. Kim, S.H. Kim, Chemical and structural modifications of RNAi therapeutics, Adv. Drug Deliv. Rev. 104 (2016) 16–28. https://doi.org/10.1016/j.addr.2015.10.015.

[37] M.F. Osborn, A. Khvorova, Improving siRNA Delivery in Vivo Through Lipid Conjugation, Nucleic Acid Ther. 28 (2018) 128–136.

https://doi.org/10.1089/nat.2018.0725.

[38] G. Ozcan, B. Ozpolat, R.L. Coleman, A.K. Sood, G. Lopez-Berestein, Preclinical and clinical development of siRNA-based therapeutics, Adv. Drug Deliv. Rev. 87 (2015) 108–119. https://doi.org/10.1016/j.addr.2015.01.007.

[39] M. Dominska, D.M. Dykxhoorn, Breaking down the barriers: siRNA delivery and endosome escape, J. Cell Sci. 123 (2010) 1183–1189. https://doi.org/10.1242/jcs.066399.

[40] S.M. Saraiva, V. Castro-López, C. Pañeda, M.J. Alonso, Synthetic nanocarriers for the delivery of polynucleotides to the eye, Eur. J. Pharm. Sci. 103 (2017) 5–18. https://doi.org/10.1016/j.ejps.2017.03.001.

[41] Y. Weng, J. Liu, S. Jin, W. Guo, X. Liang, Z. Hu, Nanotechnology-based strategies for treatment of ocular disease, Acta Pharm. Sin. B. 7 (2017) 281–291. https://doi.org/10.1016/j.apsb.2016.09.001.

[42] D.J. Bharali, I. Klejbor, E.K. Stachowiak, P. Dutta, I. Roy, N. Kaur, E.J. Bergey, P.N. Prasad, M.K. Stachowiak, Organically modified silica nanoparticles: A nonviral vector for in vivo gene delivery and expression in the brain, Proc. Natl. Acad. Sci. 102 (2005) 11539–11544. https://doi.org/10.1073/pnas.0504926102.

[43] C.E. Ashley, E.C. Carnes, K.E. Epler, D.P. Padilla, G.K. Phillips, R.E. Castillo, D.C. Wilkinson, B.S. Wilkinson, C. a Burgard, R.M. Kalinich, J.L. Townson, B. Chackerian, C.L. Willman, D.S. Peabody, W. Wharton, C.J. Brinker, Delivery of small interfering RNA by peptide-targeted mesoporous silica nanoparticle-supported lipid bilayers, ACS Nano. 6 (2012) 2174–2188. https://doi.org/10.1021/nn204102q.

[44] S. Chen, B. Ni, H. Huang, X. Chen, H. Ma, siRNA-loaded PEGylated porous silicon nanoparticles for lung cancer therapy, J. Nanoparticle Res. 16 (2014). https://doi.org/10.1007/s11051-014-2648-z.

[45] X. Li, Y. Chen, M. Wang, Y. Ma, W. Xia, H. Gu, A mesoporous silica nanoparticle - PEI - Fusogenic peptide system for siRNA delivery in cancer therapy, Biomaterials. 34 (2013) 1391–1401. https://doi.org/10.1016/j.biomaterials.2012.10.072.

[46] N. Wareing, K. Szymanski, G.R. Akkaraju, A. Loni, L.T. Canham, R. Gonzalez-Rodriguez, J.L. Coffer, In Vitro Gene Delivery with Large Porous Silicon Nanoparticles Fabricated Using Cost-Effective, Metal-Assisted Chemical Etching, Small. 13 (2017) 13–17. https://doi.org/10.1002/smll.201602739.

[47] J. Shen, X. Wu, Y. Lee, J. Wolfram, Z. Yang, Z.-W. Mao, M. Ferrari, H. Shen, Porous Silicon Microparticles for Delivery of siRNA Therapeutics, J. Vis. Exp. (2015) 1–9. https://doi.org/10.3791/52075.

[48] L.M. Jurkić, I. Cepanec, S.K. Pavelić, K. Pavelić, Biological and therapeutic effects of ortho-silicic acid and some ortho-silicic acid-releasing compounds: New perspectives for therapy, Nutr. Metab. (Lond). 10 (2013) 2. https://doi.org/10.1186/1743-7075-10-2.

[49] J. Liu, A. Stace-Naughton, C.J. Brinker, Silica nanoparticle supported lipid bilayers for gene delivery, Chem. Commun. (2009) 5100–5102. https://doi.org/10.1039/b911472f.

[50] J. Liu, A. Stace-naughton, X. Jiang, C.J. Brinker, Porous Nanoparticle Supported Lipid Bilayers (Protocells) as Delivery Vehicles, (2009) 1354–1355. https://doi.org/10.1021/ja808018y.

[51] X. Li, Q.R. Xie, J. Zhang, W. Xia, H. Gu, The packaging of siRNA within the mesoporous structure of silica nanoparticles, Biomaterials. 32 (2011) 9546–9556. https://doi.org/10.1016/j.biomaterials.2011.08.068.

[52] J. Joo, E.J. Kwon, J. Kang, M. Skalak, E.J. Anglin, A.P. Mann, E. Ruoslahti, S.N. Bhatia, M.J. Sailor, Porous silicon–graphene oxide core–shell nanoparticles for targeted delivery of siRNA to the injured brain, Nanoscale Horiz. 1 (2016) 407–414. https://doi.org/10.1039/C6NH00082G.

[53] H. Katas, H.O. Alpar, Development and characterisation of chitosan nanoparticles for siRNA delivery., J. Control. Release. 115 (2006) 216–25. https://doi.org/10.1016/j.jconrel.2006.07.021.

[54] J. Devoldere, K. Peynshaert, H. Dewitte, C. Vanhove, L. De Groef, L. Moons, S.Y. Özcan, D. Dalkara, S.C. De Smedt, K. Remaut, Non-viral delivery of chemically modified mRNA to the retina: Subretinal versus intravitreal administration, J. Control. Release. 307 (2019) 315–330. https://doi.org/10.1016/j.jconrel.2019.06.042.

[55] S. Honary, F. Zahir, Effect of zeta potential on the properties of nano-drug delivery systems - A review (Part 1), Trop. J. Pharm. Res. 12 (2013) 255–264. https://doi.org/10.4314/tjpr.v12i2.19.

[56] A. Verma, F. Stellacci, Effect of surface properties on nanoparticle-cell interactions, Small. 6 (2010) 12–21. https://doi.org/10.1002/smll.200901158.

[57] S. Honary, F. Zahir, Effect of zeta potential on the properties of nano-drug delivery systems - A review (Part 1), Trop. J. Pharm. Res. 12 (2013) 255–264. https://doi.org/10.4314/tjpr.v12i2.19.

[58] Q. Luo, J. Zhao, X. Zhang, W. Pan, Nanostructured lipid carrier (NLC) coated with Chitosan Oligosaccharides and its potential use in ocular drug delivery system, Int. J. Pharm. 403 (2011) 185–191. https://doi.org/10.1016/j.ijpharm.2010.10.013.

[59] T. Hagigit, T. Nassar, F. Behar-Cohen, G. Lambert, S. Benita, The influence of cationic lipid type on in-vitro release kinetic profiles of antisense oligonucleotide from cationic nanoemulsions, Eur. J. Pharm. Biopharm. 70 (2008) 248–259. https://doi.org/10.1016/j.ejpb.2008.03.005.

[60] J. Hu, X. Shen, F. Rigo, T.P. Prakash, V.V. Mootha, D.R. Corey, Duplex RNAs and ss-siRNAs Block RNA Foci Associated with Fuchs' Endothelial Corneal Dystrophy, Nucleic Acid Ther. 29 (2019) 73–81. https://doi.org/10.1089/nat.2018.0764.

[61] G.K. Klintworth, Corneal dystrophies, Orphanet J. Rare Dis. 4 (2009) 7. https://doi.org/10.1186/1750-1172-4-7.

[62] A.J. Aldave, B. Sonmez, S.L. Forstot, S.A. Rayner, V.S. Yellore, B.J. Glasgow, A Clinical and Histopathologic Examination of Accelerated TGFBIp Deposition After LASIK in Combined Granular-Lattice Corneal Dystrophy, Am. J. Ophthalmol. 143 (2007) 416–419. https://doi.org/10.1016/j.ajo.2006.11.056.

[63] K.E. Han, S. Choi, T. Kim, Y.-S. Maeng, R.D. Stulting, Y.W. Ji, E.K. Kim, Pathogenesis and treatments of TGFBI corneal dystrophies, Prog. Retin. Eye Res. 50 (2016) 67–88. https://doi.org/10.1016/j.preteyeres.2015.11.002.

[64] C.B.T. Moore, K.A. Christie, J. Marshall, M.A. Nesbit, Personalised genome editing - The future for corneal dystrophies, Prog. Retin. Eye Res. 65 (2018) 147–165. https://doi.org/10.1016/j.preteyeres.2018.01.004.

[65] M. Bidaut-Garnier, E. Monnet, A. Prongué, R. Montard, A.S. Gauthier, M. Desmarets, A.S. Mariet, C. Ratajczak, D. Binda, M. Saleh, B. Delbosc, Evolution of Corneal Graft Survival over a 30-Year Period and Comparison of Surgical Techniques: A Cohort Study, Am. J. Ophthalmol. 163 (2016) 59–69. https://doi.org/10.1016/j.ajo.2015.12.014.

[66] B. Seitz, W. Lisch, Stage-Related Therapy of Corneal Dystrophies, in: Corneal Dystrophies, KARGER, Basel, 2011: pp. 116–153. https://doi.org/10.1159/000324081.

[67] K.A. Christie, D.G. Courtney, L.A. DeDionisio, C.C. Shern, S. De Majumdar, L.C. Mairs, M.A. Nesbit, C.B.T. Moore, Towards personalised allele-specific CRISPR gene editing to treat autosomal dominant disorders, Sci. Rep. 7 (2017) 16174. https://doi.org/10.1038/s41598-017-16279-4.

[68] D.G. Courtney, J.E. Moore, S.D. Atkinson, E. Maurizi, E.H.A. Allen, D.M.L. Pedrioli, W.H.I. McLean, M.A. Nesbit, C.B.T. Moore, CRISPR/Cas9 DNA cleavage at SNP-

derived PAM enables both in vitro and in vivo KRT12 mutation-specific targeting, Gene Ther. 23 (2016) 108–112. https://doi.org/10.1038/gt.2015.82.

[69] Y. Taketani, K. Kitamoto, T. Sakisaka, M. Kimakura, T. Toyono, S. Yamagami, S. Amano, M. Kuroda, T. Moore, T. Usui, Y. Ouchi, Repair of the TGFBI gene in human corneal keratocytes derived from a granular corneal dystrophy patient via CRISPR/Cas9-induced homology-directed repair, Sci. Rep. 7 (2017) 16713. https://doi.org/10.1038/s41598-017-16308-2.